SEROLOGY

MEDICAL LABORATORY TECHNIQUES
FOR ROUTINE DIAGNOSTIC TESTS

Volume X

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G. Guru
Project Coordinator

राष्ट्रीय शैक्षिक अनुसंधान और प्रशिक्षण परिषद्
NATIONAL COUNCIL OF EDUCATIONAL RESEARCH AND TRAINING

October 1990
Kartika 1912

P.D 5T — RA

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Rs. 3 – 50

Published at the Publication Department by the Secretary, National Council of Educational Research and Training, Sri Aurobindo Marg, New Delhi 110016, laser typeset by M/s Compugraphics, H-16, Green Park Extension, New Delhi 110016 and printed at M/s A.J. Printers, 5 Pratap Bhawan, Bahadur Shah Zafar Marg, New Delhi 110002.

PREFACE

Ever since the introduction of vocationalization of education in our school system by several States and Union Territories in our country, the paucity of appropriate instructional materials has been felt as one of the major constraints in the implementation of the programme and a source of great hardship to pupils offering vocational studies at the higher secondary stage.

The Department of Vocationalization of Education of the National Council of Educational Research and Training, New Delhi, has started a modest programme of developing instructional materials of diverse types to fill this void in all major areas of vocational education. The task is too gigantic to be completed by any single agency but the model materials being developed by us might provide guidance and impetus to the authors and agencies desiring to contribute in this area. These are based on to the national guidelines developed by a working group of experts constituted by the NCERT.

The present volume is on Serology and is meant for the pupils and teachers of the Medical Laboratory Technician vocation course being offered in a number of States and Union Territories. It is hoped that the users will find it immensely useful.

The material was developed during a workshop held in the Dr. T.M.A. Pai Research Centre, Manipal. The present version was finalized after incorporating suggestions and comments. The names of the contributors, reviewers are mentioned elsewhere and their contributions are admirably acknowledged. Shri G. Guru, Reader and Coordinator of the project, Department of Vocationalization of Education, deserves special thanks for technical editing and for bringing the material into the present form. The assistance of all in the Dr. T.M.A. Pai Research Centre, especially, Dr. A. Krishna Rao, Dean, Kasturba Medical College, Manipal, and that of the Department of Vocationalization of Education is also gratefully acknowledged.

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FOREWORD

The programme of vocationalization of higher secondary education has been accepted by the country as it holds forth great promise for linking education with the productivity and economic development of the country by providing education for better employability of the youth.

In view of the importance of the programme, the NCERT is making an all out effort to provide academic support to the implementing agencies in the States. One of the major contributions of the NCERT is in the field of curriculum development and in the development of model instructional materials. The materials are developed through workshops in which experts, subject specialists, employers' representatives, curriculum framers and teachers of the vocational course are involved.

The present volume on *Serology* is one of the series on "Medical Laboratory Techniques for Routine diagnostic Tests" and is meant for the students of the Medical Laboratory Technician Course. It is being published for wider dissemination amongst students and teachers throughout the country. I hope that they will find the volume useful.

I am grateful to those who have contributed to the development of this volume. I must acknowledge also the immense interest taken by Prof. A.K. Mishra, Head, Department of Vocationalization of Education, in inspiring his colleagues in their endeavours to develop instructional materials. Shri G. Guru, Reader, functioned as the Project Coordinator for the development of this title. He has my appreciation and thanks for planning, designing and conducting the workshop, for technical editing and for seeing this title through the press.

Suggestions for improvement of this volume will be welcome.

K. GOPALAN
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ACKNOWLEDGEMENTS

The participation of the following expert in the development of this title as contributor/reviewer is gratefully acknowledged.

Contributor/Reviewer

Dr. P.G. Sivananda

GANDHIJI'S TALISMAN

"I will give you a talisman. Whenever you are in doubt or when the self becomes too much with you, apply the following test :

Recall the face of the poorest and the weakest man whom you may have seen and ask yourself if the step you contemplate is going to be of any use to him. Will he gain anything by it ? Will it restore him to a control over his own life and destiny ? In other words, will it lead to Swaraj for the hungry and spiritually starving millions ?

Then you will find your doubts and your self melting away."

mkGandhi

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Chapter 1

Introduction

1.1 Definition, Principles and Scope of Serology

Serology is the study, in vitro, of certain properties of serum, specifically, the reactions between antigens and antibodies. Serology is not synonymous with immunology which includes many factors of resistance other than those associated with the serum reactions. Serological tests are used for various purposes :

(a) To find out an unknown antigen and antibody.
(b) For quantitative estimation of antibodies.
(c) For the diagnosis of infectious diseases.
(d) For the standardization of toxins and antitoxins.
(e) In the determination of blood groups and in blood transfusion.
(f) In forensic medicine.
(g) In the study of allergy, hypersensitivity, autoimmune diseases and tissue transplantation.

1.2 Collection of Blood

About 5 ml of blood is collected in a dry sterile tube using a dry sterile syringe. Blood anticoagulants should not be used. After collection, blood has to be kept at room temperature for one hour. Then the clot is dislodged using a sterile loop and refrigerated for 3-4 hours; during this period, the serum exudes and the clot retracts. The serum is aspirated by using a sterile pasteur pipette after centrifugation. The serum thus obtained must be clear and free from haemolysis. Collection of paired sera during the acute as well as convalescent period of an illness is preferred to demonstrate rise in antibody titre.

1.3 Antigen and Antibody

An antigen is usually a foreign substance which, when it enters the body, stimulates the production of an antibody with which it reacts specifically in an observable manner.

Antibodies are the proteins which appear in the serum and tissue fluids of the host in response to antigenic stimuli.

Antigens and antibodies combine with each other specifically in an observable manner and such reactions serve several diagnostic purposes.

1.4 Antigen and Antibody Reactions

(a) Agglutination

This is one of the widely employed serological tests. In this test, the particulate antigen when mixed with the serum containing corresponding antibodies results in clumping or agglutination. Agglutination tests are of two types :

(i) Slide agglutination test used for:
  - — Identification of *Salmonella* and *Shigella*.
  - — Typing of *Pneumococci*.
  - — Griffith's typing of *Streptococci*.

(ii) Tube agglutination test are :
  - (1) Widal test.
  - (2) Brucella agglutination test.
  - (3) Weil Felix test.
  - (4) Rose Waaler test.
  - (5) Cold haemagglutination test.
  - (6) Streptococcus MG agglutination test.
  - (7) Paul-Bunnell test.

(b) *Precipitation*

When a soluble antigen combines with its specific antibody in the presence of electrolytes at a suitable temperature and pH, there will be formation of an insoluble precipitate of antigen-antibody complex. This phenomenon is called precipitation. When, instead of sedimenting, the precipitate remains suspended as floccules, the reaction is known as *FLOCCULATION*. Precipitation can take place in liquid media and in gels as well.

Example: 1. Ring test.
2. Slide test.
3. Tube test.
4. Immunodiffusion.

(c) *Complement Fixation*

Complement is a substance present in normal serum which gets activated characteristically by antigen-antibody interaction. The ability of antigen-antibody complexes to 'fix' the complement is made use of in the complement fixation test (CFT). In the presence of appropriate antibodies, the complement lyses erythrocytes, kills bacteria and immobilizes motile organisms, etc. CFT is a highly sensitive test used in the diagnosis of several microbial diseases including syphilis.

Chapter 2

Widal Test

This is tube agglutination test done for the diagnosis of enteric fever. The test is based on the demonstration of somatic and flagellar antibodies to S. typhi/ S. Paratyphi A in the sera of patients suffering from enteric fever.

2.1 Requirements

(a) Felix and Dreyer's tubes.
(b) Bacteriological saline.
(c) 'O' and 'H' antigens.
(d) Test serum.
(e) Pipettes and racks.
(f) Waterbath.

2.2 Preparation of Antigen

2.2.1. Somatic or 'O' antigen

1. The standard, non-motile 901 'O' strain of Salmonella typhi is inoculated into nutrient broth and incubated at 37°c for 6-8 hours.
2. A nutrient agar slope in Roux bottle (prechecked for sterility) is

inoculated with this broth culture and the slope is incubated at 37°c for 24-48 hours.

3. The growth on the slope is 'scraped' with minimum quantity of bacteriological saline and about 20 times the volume of absolute alcohol is added to this antigenic suspension.
4. The suspension is heated in a waterbath at 50° - 55°c for one hour.
5. Alcohol is removed by centrifugation and the residual 'O' antigen is resuspended in bacteriological saline.
6. The antigen is finally standardized by adjusting to a suitable BROWN'S OPACITY TUBE.
7. Before releasing for routine use, 'O' antigen is checked for sterility and potency using known positive and negative control sera.

2.2.2 *Flagellar or 'H' Antigen*

1. The standard, motile 901 'H' strain of S.typh./highly motile strain of S. paratyphi A° is inoculated into nutrient broth and incubated at 37°c for 6-8 hours.
2. A nutrient agar slope in Roux bottle (prechecked for sterility is inoculated with this broth culture and the slope is incubated at 37°c for 24-48 hours.
3. The growth on the slope is 'scraped' with minimum quantity of saline.
4. This 'H' antigenic suspension is then standardized with 0.2% formal saline using a suitable BROWN'S OPACITY TUBE.
5. Before routine use, 'H' antigen is checked for sterility and potency.

2.3 Procedure

*2.3.1. 'O' Agglutination (*Using *FELIX TUBES)*

1. Arrange 8 Felix tubes (round bottomed) in a rack. Tubes 1 to 7 are used for test dilution, the 8th tube serves as antigen control.
2. To a separate sterile test tube, add 0.5 ml of the 1/10 test serum and 4.5 ml of 0.85% saline. This gives 1 : 10 dilution of the serum.
3. Add 0.5 ml 0.85% saline to tubes 2 to 8.
4. With a fresh graduated 1 ml pipette, add 0.5 ml of the 1 : 10 diluted serum to each of the tubes 1 and 2.

Tube 1 contains 0.5 ml of 1 : 10 diluted serum only.

Tube 2 contains 0.5 ml saline + 0.5 ml of 1 : 10 diluted serum.

5. Mix the fluid in tube 2 by pipetting up and down several times to mix; then transfer 0.5 ml to tube 3. Repeat this process to tube 7, from which discard 0.5 ml.
6. Each tube now contains 0.5 ml fluid, the serum dilutions in tubes 1 to 7 being 10, 20, 40, 80, 160, 320 and 640 respectively. Tube 8 contains only 0.5 ml saline.
7. With fresh 1 ml pipette, add 0.5 ml of bacterial suspension to all the 8 tubes. Now the serum dilutions in tubes 1 to 7 are 20, 40, 80, 160, 320, 640 and 1280 respectively.
8. Incubate the tubes in a waterbath at 37°c for 4 to 6 hours and read after overnight refrigeration at 4°c. Read the results using a microscopic mirror or a magnifying lens.

Result: The titre of serum is read as the greatest dilution giving compact, granular, chalky agglutination. A well formed bottom will be formed at the bottom of the control tube indicating no agglutination.

2.3.2. *'H' Agglutination* (DREYER'S METHOD)

In a suitable rack, place a row of 5 Dreyer's agglutination tubes and a test tube (3×3/8") which may be called the dilution tube. Into this tube add 18 drops of normal saline (0.85%) measuring with the dropping pipette; similarly, add 2 drops of serum and mix. This yields 1 : 10 serum dilution; saline, serum dilutions and bacterial suspension are now added to the 5 agglutination tubes as follows:

Tubes	1	2	3	4	5
Saline	0	5	8	9	10
Serum 1 :	10	10	5	2	10
Bacterial Suspension	15	15	15	15	15
Final dilution of serum	1 : 25	1 : 50	1 : 125	1 : 250	control.

Incubate the tubes in a waterbath at 50-55°c for 2 hours, or at 37°c for 4 hours. Allow to settle and read the results using a microscopic mirror.

Result : 'H' agglutination is characterised by loose, large, fluffy clumps.

Chapter 3

VDRL Test

This is slide or tube flocculation test done for the diagnosis of syphilis.

3.1 Procedure

3.1.1 Antigen preparation

In a stoppered bottle, place 0.4 ml of the buffered saline and add to it, drop by drop, from a pipette, 0.5 ml of antigen. Ensure that the antigen is added during a period of approximately 6 seconds and that the bottle is continuously rotated during this time. After the addition, the bottle is rotated vigorously for a further 10 seconds. Now add 4.1 ml of 1% buffered saline. Gently shake about 30 times in 10 seconds and allow to stand for 5 minutes. The antigen is now ready and can be used during the next 8 hours.

3.1.2 Preparation of Test Sera

Clear sera are heated at 56°c for 30 minutes. Sera containing particulate debris are recentrifuged.

3.1.3 Qualitative Serum Test

1. 0.05 ml of inactivated serum is taken in one ring of a paraffin ringed slide.
2. One drop of freshly prepared antigen is added with a syringe, delivering 60 drops to the ml.
3. Positive and negative serum controls + an antigen control is also included.
4. The slide is rotated at 120 rotations per minute in a VDRL rotator for 4 minutes.
5. It is then examined immediately under the microscope with low power objective. Observe the controls first.

Result :		
	Uniform distribution of crystals :	Non-reactive
	Formation of small clumps :	Weak reactive
	Formation of large clumps :	Strongly reactive

3.1.4 Quantitative Serum Test

These tests are performed on serially diluted sera in saline, each dilution being treated as an individual serum. Different serum dilutions are prepared in 0.9% saline as follows :

1. Place 0.5 ml of saline in each of 6 or more tubes.
2. Add 0.5 ml of heated serum to tube 1; mix well and transfer 0.5 ml from this tube (tube 1) to tube 2.

Serum dilutions

1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	Report
++	++	++	++	+	-	1 : 16 dilution or 16 dil
++	+	-	-	-	-	1 : 2 dilution or 2 dil
+	-	-	-	-	-	Reactive undiluted 1 dil

3. Continue this operation till the 6th or last tube (which contains 1 ml). So the dilutions in these tubes will be 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32, 1 : 64 etc.
4. Place 0.05 ml of different dilutions of serum on different paraffin rings on the slide.
5. Add 1 drop (1/60 ml) of antigen emulsion and rotate the VDRL slide for 4 minutes on the VDRL rotator and read immediately.

Result: Report the results in terms of the greatest serum dilution that produces a positive reaction.

Chapter 4

Spinal Fluid Test

4.1 Qualitative Spinal Fluid Test

Procedure

1. Prepare the antigen emulsion as in the qualitative serum test. Add 1 part of 10% sodium chloride solution to 1 part of the antigen emulsion. Mix well and allow to stand at least 5 minutes, but not more than 2 hours, before use.
2. Pipette 1 ml of heated spinal fluid into a 1 : 3×10 cm test tube. Include positive and negative spinal fluid controls.
3. Shake the antigen container well and add 0.2 ml to each spinal fluid (1 ml).
4. Shake racks of tubes on Kahn shaking machine for 15 minutes.
5. Centrifuge all the tubes at 1800 r.p.m. for 5 minutes. Return the tubes to Kahn shaker and shake for exactly 2 minutes.
6. Read reactions as soon as possible by holding the tubes close to the shade of a desk lamp having a black background.
   Avoid excessive agitation.

Result :

(a) Definitely visible aggregates suspended in clear water or turbid medium : Reactive.

(b) No aggregation and complete dispersion of particles with turbid or slightly granular appearance : Non-reactive

4.2 Quantitative Spinal Fluid Test

1. Preparation of spinal fluid dilutions :
   - (a) Pipette 1 ml of 0.9% Nacl solution into each of 5 or more tubes.
   - (b) Add 1 ml of heated spinal fluid to tube 1; mix well and transfer 1 ml to tube 2.
   - (c) Continue mixing and transferring till the last tube, from which discard 1 ml. The dilutions are 1 : 2, 1 : 4, 1 : 8, 1 : 16, 1 : 32 etc.
2. Add 0.2 ml of sensitized antigen to each tube and shake the tubes in a Kahn shaker for 15 minutes; centrifuge for 5 minutes and shake again for 2 minutes in the Kahn shaker.
3. Read the results as in the qualitative spinal fluid test.

Result : Report the results in terms of highest dilution of spinal fluid which produces a positive reaction. The term "Dilutions" is employed to express the result.

Chapter 5

Weil-Felix Test

It is a heterophile tube agglutination test done for the serodiagnosis of typhus fevers. The basis of the test is the sharing of a common antigen between typhus rickettsiae and some strains of proteus bacilli i.e. *Pr. vulgaris* OX 19 and OX 2 and *Pr. mirabilis* OX K.

5.1 Requirements

(a) Patient's serum, inactivated at 45°c for 30 minutes.
(b) *Proteus* OX 19, OX 2 and OX K antigenic suspensions.
(c) 3×1/2 inch tubes.
(d) Saline solution.
(e) Rack, pipettes and 37°c waterbath.

5.2 Procedure

(1) Set up 3 rows of 7 tubes each, the 7th tube labelled as control.
(2) To the first 6 tubes in each row, add 1 ml each respectively of 1/20, 1/40, 1/80, 1/160, 1/320, and 1/640 diluted patients serum. To the control tubes, add 1 ml of saline only.
(3) One drop of concentrated *Proteus* OX 19 suspension is added to all the 7 tubes in the first row, one drop of *Proteus* OX 2 suspension to 7 tubes in the second row and one drop of *Proteus* OX K suspension to 7 tubes in the third row.

(4) Incubate the tubes at 37°c in a waterbath for 2 or 3 hours and read.

5.3 Results: The highest serum dilution showing visible agglutination is expressed as the antibody titre of the serum. A positive agglutination with OX 19 is indicative of classical epidemic typhus, while a positive test with OX 2 suspension indicates murine typhus and that with OX K suspension indicates scrub typhus.

Chapter 6

Antistreptolysin 'O' Titration

It is a quantitative toxin neutralization test which is used as a laboratory aid in the diagnosis of group A streptococcal infections, notably, rheumatic fever and acute glomerulonephritis. Due to Group A streptococcal infection, there will be appearance of a corresponding antibody-antistreptolysin O in the patient's serum. Haemolysis of red cells by streptolysin O can be inhibited by this antibody. For determination of antistreptolysin O titration, a constant amount of streptolysin O is added to decreasing amount of serum; if the antistreptolysin present is sufficient to neutralize the antigen, no haemolysis occurs when red cells are subsequently added. A point is reached where the antigen exceeds the antibody and the excess of streptolysin 'O' causes haemolysis of the added red cells.

6.1 Requirements

(a) Streptolysin O reagent (SLO).
(b) Antistreptolysin O control serum.
(c) Streptolysin O buffer.
(d) 5% rabbit or human group 'O' RBC suspension.
(e) Small test tubes.
(f) Pipettes, racks, waterbath — 37°c

6.2 Procedure

Pre-dilutions	1 : 10		1 : 100					1 : 500					*RBC Control*	*SLO Control*
	1	2	3	4	5	6	7	8	9	10	11	12		
Serum pre-dilution in ml	0.8	0.2	1	0.8	0.6	0.4	0.3	1	0.8	0.6	0.4	0.2	0	0
Buffer in ml	0.2	0.8	0	0.2	0.4	0.6	0.7	0	0.2	0.4	0.6	0.8	1.5	1
SHAKE GENTLY AND MIX														
SLO reagent in ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0	0.5
SHAKE GENTLY TO MIX AND INCUBATE AT 37°C FOR 10 MINUTES														
5% rabbit or human 'O' RBC suspension	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Shake Gently to mix, incubate at 37°c for 30 minutes (after 15 minutes, shake tubes again) after incubation, shake tubes again and read														
Todd Units	12	50	100	125	166	250	333	500	625	833	1250	2500		

6.3 Result : The Antistreptolysin 'O' titre is the reciprocal or the highest serum dilution that prevents haemolysis of the cells. It is expressed in terms of TODD UNITS.

Chapter 8

'C' Reactive Protein (CRP) Test

It is a rapid, slide latex agglutination test used for the detection of C-reactive protein in human serum. CRP usually appears in the sera of patients in the acute stages of a number of inflammatory conditions—most bacterial and some viral infections i.e. acute rheumatic fever with or without carditis, rheumatoid arthritis and most of the other collagen diseases and in a number of other conditions characterized by inflammation. It can usually be demonstrated in cases of acute myocardial infarction and in several types of malignancies, particularly those with metastasis.

The test is dependent on the immunologic reaction between CRP as an antigen and the corresponding antibody coated on the surface of biologically inert latex particles.

8.1 Requirements

(a) Latex CRP reagent.
(b) Positive and negative control sera.
(c) Disposable plastic droppers, applicator sticks and slide.

8.2 Procedure

Note : Include a positive and negative serum control with every test.

8.3 Result : Agglutination of latex particles indicates a positive test.

Chapter 9

Pregnancy Test

The slide test for pregnancy is a test done for diagnosis of pregnancy. The test is based on the principle of agglutination inhibition. The human urine to be tested is mixed with serum containing Human Chorionic Gonadatrophin (HCG) antibody and latex particles to which the HCG antigen is bound. In the case of a non-pregnant female, the urine does not normally contain HCG so that the antibody is free to react with the HCG bound to the latex. The result is agglutination indicating a negative test (not pregnant). In the case of a pregnant female, the urine contains HCG which reacts to neutralize or inhibit the antibody from reacting with the HCG bound to the latex; the result is no agglutination, indicating a positive test (pregnant).

9.1 Requirements

(a) Anti-HCG serum.
(b) Suspension of latex particles coated with HCG.
(c) Black glass slides.
(d) Disposable plastic pipettes and mixing sticks.
(e) Urine test samples, collected preferably in the morning.

9.2 Procedure

1. Bring reagents and urine to room temperature.

Chapter 7

Latex Fixation Test for Rheumatoid Factor

It is a passive slide agglutination test done for detection of RHEUMATOID FACTOR (RF) in serum of suspected cases of rheumatoid arthritis. The antigen consists of polystyrene latex particles coated with specially purified human gamma globulin. The suspension of coated latex particles agglutinate visibly when mixed with a diluted serum containing rheumatoid factor.

7.1 Requirements

(a) Latex gamma globulin reagent.
(b) Glycine buffer.
(c) Positive and negative control sera.
(d) Slide, disposable plastic droppers and applicator sticks, tubes.

7.2 Procedure

1. Allow the reagents and serum samples to attain room temperature.
2. In a tube, dispense 0.95 ml of glycine buffer, add 0.05 ml of test serum and mix.
3. Using the disposable plastic dropper, place one drop of diluted test serum within the circled area marked TEST on the special slide

provided in the kit.

4. Add 1 drop of latex gamma globulin reagent to the above drop after mixing the contents of the vial.
5. Mix well with a disposable applicator stick and rock the slide gently to and fro for 2 minutes and examine for agglutination.

Note : A positive and negative controls must be included with every test.

7.3 Result: Coarse agglutination usually occurring within 1 minute is considered STRONG POSITIVE whereas finer agglutination usually taking full 2 minutes is considered WEAK POSITIVE. A negative test is indicated by a smooth suspension with no obvious change.

2. With the disposable pipette provided, place one drop of urine on lower portion of one circle of the black glass slide.
3. Add a drop of anti-HCG serum to the urine drop on the slide.
4. Place 2 drops of well-mixed antigen on the upper half of the same square of the black glass slide.
5. With the disposable mixing stick provided, mix anti-HCG serum and urine at least 10 times, avoiding the antigen. Then blend in antigen, spreading mixture over the entire square.
6. Tilt slowly and gently for 1-2 minutes while observing for agglutination.

Note : Include known positive and negative urines for validating the reagents and the procedure.

9.3 Results: Absence of agglutination indicates a positive test or pregnancy and presence of agglutination indicates a negative test.

Chapter 10

Mantoux Test

It is categorized under immunological bacterial skin tests. It is one of the methods of tuberculin testing used as an aid in diagnosing active infection in infants and young children, to measure the prevalence of infection in a community, to select susceptibles for vaccination or as an indication of successful vaccination.

10.1 Procedure

The test is performed by intradermal injection on the forearm, of 0.1 ml of a 1/10,000 dilution (ITU) of tuberculin (PPD). If there is no reaction, concentrations of 1/1000 and 1/100 are used. Reading is made at the end of 48 hours.

10.2 Observation

An area of odema and induration, atleast 8 mm in diameter appearing at the site of injection indicates a positive test. Erythema alone is not taken as a positive reaction.

Appendix

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